THE RELATION OF FAT CHANGES TO THE GENERAL CHEMICAL EMBRYOLOGY OF THE SEA URCHIN

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Introduction

Several years ago (Hayes, 1934) a study was made of the nitrogen metabolism in the early egg stages of the Florida sea urchin, *Echinometra lucunter*. In the previous year Ephrussi published a general account of the metabolism of developing *Paracentrotus* eggs. The papers agreed in finding a loss of nitrogen during later development; for the period immediately following fertilization there are no data in the latter paper, and in the former the results suggested an increase in nitrogen which, since such a state of affairs is otherwise unknown in embryology, was treated with caution pending confirmation. Nitrogen then, and therefore protein, is evidently a source of energy in later development, probably from the eight-hour hatching stage on.

This paper records the results of a study of another possible source of developmental energy, namely fat. Ephrussi (1933) offers the only earlier study, and he found a diminution of fat as development proceeded. Since he tested only three ages, namely unfertilized, 12 hours and 40 hours, his results do not show the precise time at which the loss took place, and cannot be used to supplement and check those to be presented below.

Ephrussi expresses his results as percentages of the wet or dry weight of the egg, a procedure which can be standardized when one is dealing with unfertilized eggs alone, but which presents great difficulties when it is necessary to take into consideration the changes in volume and shape which occur during development. Eggs usually have to be concentrated for analysis by centrifugation, and the number of eggs which will occupy 1 ml. in a centrifuge tube at a given speed is not the same at any two ages. If eggs are sectioned and examined it is possible to derive formulae for the estimation of volume at different stages (Pelluet, 1938). If the volumes are now combined with counts showing how many eggs can be packed into 1 ml., data are at hand for the expression of any egg constituent as a percentage, e.g. fat. This procedure would be very tedious; has not been carried out by any

investigator; and if it were would not be profitable because the truth would be obscured by fluctuations in the water content which would make e.g. a stationary fat concentration per egg appear to vary. For these reasons and others which have been discussed at length (Hayes, 1934), the values below are given per million eggs rather than in per cent.

Methods

The first requirement in work of this kind is a method for rearing eggs in large numbers, free from debris and bacteria, and showing a high percentage of normal development. Attempts were made at first to work with starfish eggs, but the last condition could not be satisfactorily met, and *Arbacia* was substituted. Eggs were obtained from several females, fertilized and reared in finger bowls according to the well-known methods devised by Just and others. All stages were examined to ascertain the percentage developing normally and it was always over 90.

When it was desired to collect a sample the eggs were concentrated in centrifuge tubes at low speeds. This procedure could not be used for swimming stages, which were collected by filtration through fine bolting silk. Following either treatment, eggs or larvae were transferred to a volumetric flask of sea water, usually of one liter capacity, and the water made up to the mark. Water and eggs were then transferred to a larger vessel and thoroughly mixed. While the mixing was continued 1 ml. samples were withdrawn with a brass Stempel pipette and transferred to a Sedgwick-Rafter plankton counting slide of capacity 1 ml., provided with a cover slip. (I am indebted to Dr. C. J. Fish for the loan of a Stempel pipette and counting slide.) Ten fields of the slide, selected at random, were then counted with a microscope whose lenses had previously been calibrated with a stage micrometer. The total was added up and constituted one observation. Usually four such observations were made on each of two samples and the results averaged. Two observers alternated on the counting in order to eliminate the personal factor as far as possible. The probable errors in counting ranged from $\pm$ 0.39 per cent to $\pm$ 10.2 per cent with an average value of $\pm$ 4.1 per cent.

After the counts were made the eggs were again concentrated in centrifuge tubes, and transferred from these to a 100 ml. volumetric flask. The fats were extracted by the wet alcohol-ether method described by Fowweather (1926), the flask was made up to the mark, and the fat-containing extract filtered off and stored in glass-stoppered bottles, evaporation being prevented by sealing the tops of the bottles

with several coats of silica (water glass). When the analyses were made some months later, portions of the alcohol-ether extract were dried in a partial vacuum at room temperature and re-extracted with petroleum ether. These were made up to known volume and stored in cork-stoppered bottles, sealed by silica.

The total fat or phospholipid or sterol digitonide, was estimated by a micro-method devised by Backlin in 1930, described by Peters and Van Slyke in 1932, and slightly modified by Van Slyke, Page and Kirk (1933). Carbon dioxide, produced by the combustion of a fat sample in a special test tube attached to a Van Slyke volumetric or manometric apparatus, is carried over and reacts with dilute NaOH in the chamber, being subsequently liberated by the addition of excess

TABLE I

Variations during development in total fat, sterol and phospholipid per million eggs.

1 Age in hours and minutes	2 Total fat, mg. per million	3 Sterol, mg. per million	4 Phospholipid, mg. per million
Unfert.	5.65	0.430	2.57
1.	3.98	0.413	2.53
4.40	3.50	—	—
6.30	3.36	0.413	3.3
8.50	3.04	—	0.81
10.50	3.60	—	2.04
15.35	3.80	—	2.48
19.30	3.92	0.431	—
23.15	3.84	—	3.46
24.50	4.29	—	—
25.25	3.25	—	1.2
43.10	2.11	0.416	—

lactic acid, and measured as a gas. The probable errors resulting from extractions and fat determinations are less than $\pm$ 2 per cent. For conversion of the CO_2 into fat or into sterol the factors are given in Peters and Van Slyke, page 437. A similar table was constructed for phospholipids, based on data in Bloor (1929).

Sterol was precipitated with digitonin directly from an aliquot of the original alcohol-ether extract in one of the combustion tubes. The precipitate was washed with ether and with water, supernatent fluid being removed by means of suction through an alundum filter stick as described by Kirk, Page and Van Slyke (1934). After drying, the sterol digitonide was oxidized to CO_2 as described above. The method was checked and found to give theoretical values with cholesterol.

Total Fat

By total fat is meant all the material soluble in petroleum ether. Its variations are given in Table I, column 2 and in Fig. 1. It will be noted that from an initial value of 5.65 mg. per million in the unfertilized egg, there is a steady drop until by 9 hours there are only 3.04 mg. From this time until 20–25 hours an increase is noted, followed by a drop to the final stage observed. The most interesting points are that the trough of the curve coincides with the time of hatching, and that there is evidence of fat synthesis from approximately 9 to 23 hours. The shedding of the shell and the beginning of fat synthesis may well be associated with one another. No previous

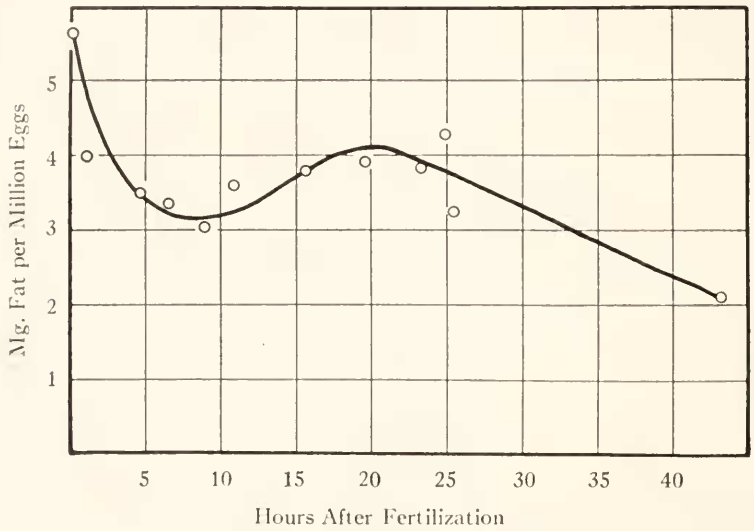

Fig. 1. Fluctuations in total fat during development. Note that the time of hatching, 8 hours, corresponds to the initial trough of the curve.

reference to the manufacture of fat by invertebrates has been found, although the phenomenon is not unknown in teleost embryos (Hayes and Ross, 1936). Since the embryo has at this time no organs of digestion, the source of the new fat is presumably some other material in the egg. There is no proof as to what this is, but a hint is obtained from the nitrogen metabolism of the Florida sea urchin, *Echinometra* (Hayes, 1934), in which, at about the time of hatching a decline in the non-amino nitrogen began, which was practically arrested when the observations closed at 24 hours. (The amino nitrogen, on the other hand, did not begin to decline until the sixteenth hour.)

Ephrussi's three values for total fat were as follows, given in each case as percentage of the wet weight.

Unfertilized	4.81
12 hours	4.43
40 hours	3.69

A glance at Fig. 1 will show that the peak at 20 hours will be missed by the particular stages selected by Ephrussi, so that his gentle decline is in agreement with Fig. 1.

Three further pieces of evidence may be mentioned, after which it will be possible to present a provisional picture of the general chemistry of early development. Warburg (1915) found in *Strongylocentrotus* a sudden burst of respiratory activity at fertilization, followed by a steady increase as development proceeded. The respiratory quotient over the first three hours was 0.9.

Secondly Runnström (1933) observed in *Paracentrotus* a sudden production of acid at fertilization. It was not carbonic acid—in fact the pH of the egg was altered to such an extent that considerable quantities of CO_2 were driven off. The consequence was that Runnström, who was making an investigation of the changes in respiration during the first hour, found that the excess CO_2 caused the apparent R.Q. for the first 10 minutes to soar to 2.37. Within half an hour the mysterious acid was being used up, and respiratory CO_2 consequently held in the egg as bicarbonate, with the result that the apparent R.Q. for 30–45 minutes was only 0.45. Although Runnström did not discover what the acid in question was, he showed that it was not phosphoric acid, and he cited the work of Perlzweig and Barron on *Arbacia* to prove that it was not lactic acid. The inference is that carbohydrate metabolism is not responsible. It may be noted here for future reference that Page (1927) found that the oil of *Arbacia* eggs had a saponification value of 606; of *Asterias* eggs 319. The oils also yielded large quantities of volatile fatty acid when distilled with steam. Fatty acids from such oils would have a low molecular weight, high solubility in water, and adequate strength to drive out CO_2.

Finally, evidence has been accumulating for some years which suggests that two kinds of respiration go on simultaneously in the egg (see Loeb and Wasteneys, 1911). It is not improbable that two substrates are burnt and that two enzymes facilitate the oxidations. That one of these belongs to the general group of oxidases, peroxidases and catalase is shown by the fact that the respiration both of fertilized and unfertilized eggs is stimulated by dimethylparaphenylene diamine (Örström, 1932). This reagent is absorbed by the eggs and becomes

in effect an artificial substrate, the measure of whose oxygen requirement is an index of the available excess enzyme in the egg. The enzyme system then, is present before fertilization, but of course the amount of oxidation which it can bring about is strictly limited by the quantity of available substrate. A second characteristic of the oxidase type of enzyme is its susceptibility to cyanide poisoning. Now cyanide has very little effect on the respiration of the unfertilized egg (Runnström, 1930), but it produces a marked inhibition of oxygen consumption after fertilization. The conclusion is that there is an abundance of enzyme, but practically no substrate before fertilization, and a small but steady supply after fertilization, which is used up as produced. As to the second part of respiration (not inhibited by cyanide) it was found that it could be stopped by narcotics, e.g. lithium (Lindahl, 1934), and stimulated by pyocyanine (Runnström, 1935*a*, both fertilized and unfertilized eggs), and by methylene blue (tried on unfertilized eggs only by Barron, 1929). Thus it passed the tests for a dehydrogenase system. Pyocyanine acts as an artificial enzyme, and the stimulus caused by its presence meant that there must have been some extra substrate there to be burnt up; in other words (in contrast with the first part) the substrate was plentiful and the small quantity of enzyme was the limiting factor.

Taking all these facts into consideration, assuming that the various sea urchins resemble one another in the essential features of their embryonic metabolism, and remembering that the times of hatching approximately coincide, the following general hypothesis of echinoid chemical embryology may be advanced.

(1) At the time of fertilization a considerable quantity of fat is split into fatty acid and glycerol, presumably by means of a lipase, the activation of which is the first of the chain of events initiating embryonic metabolism. It would be tempting to believe that the lipase is brought in by the spermatozoön, but no evidence has been found bearing on this point. For every molecule of glycerol produced there are three molecules of fatty acid; and for every calorie of energy available from glycerol there are 5 or 10 calories available from a lower fatty acid. The glycerol is scarce, the acid plentiful.

(2) The glycerol is rapidly oxidized by a cyanide-sensitive enzyme. This part of respiration does not increase as development proceeds. The enzyme was there before fertilization, but was inactive because of the absence of suitable substrate. The destruction of glycerol accounts in part for the increase of activity found at fertilization by Warburg. Glycerol continues to be produced in small quantities up to at least 8 hours, but is oxidized as formed. That cell division is

the part of development presided over by cyanide-sensitive respiration was shown many years ago by Loeb and Wasteneys (1911), who found that the concentration of cyanide exactly necessary to block development reduced the oxygen consumption to one-quarter its normal value, an amount which coincided with the normal requirement of unfertilized eggs. Runnström (1935*a*) has recently confirmed this experiment, and extended it by immersing eggs in a mixture of cyanide and pyocyanine at the same time, thereby inhibiting one part of respiration and stimulating the other. The net result was an increase in oxygen consumption, but a blocking of cell division in early prophase.

(3) Runnström's unknown acid is fatty acid. It is oxidized by the second, or cyanide-insensitive dehydrogenase system. The activities resulting from this energy source are presumably growth and basal metabolism. The enzyme does not oxidize all its substrate at once—if it did the egg would burn out in a very short time. Nevertheless, this part of respiration is shown by experiment to increase in intensity as development proceeds. An explanation of the paradox of how a limited quantity of enzyme can be made to do more work later in development than at the beginning is obvious from the work of Spek (1934), who showed that in *Asterias* and *Arbacia* eggs acid is not distributed evenly throughout the whole egg, but is strictly localised. Before fertilization the surface is acid, the interior alkaline; in early developmental stages there is a thin acid layer surrounding the dividing cells, and from the 64-cell stage to the gastrula there is a gradient of intensity of acid reaction between the animal and vegetal poles. The chemical geography of the egg limits the extent to which enzyme and substrate are able to come together. This conception is strengthened by Runnström's (1935*b*) statement that "All our experimental evidence indicates that the susceptibility to the action of lithium is highest at the animal pole and decreases gradually."

(4) Fat ceases to be a source of energy at approximately the time of hatching, 8 hours, possibly because it is required as building material.

(5) From hatching time up to 16 hours energy is provided by non-amino nitrogenous materials (non-protein?), from which fat is synthesized and possibly the costs of cell division, motion and basal metabolism are in part or in whole met.

(6) To the utilization of non-amino nitrogenous materials there is added at 16 hours, a gradual destruction of compounds containing amino nitrogen as well.

(7) At 24 hours fat begins once again to decline while at the same time the destruction of nitrogenous material appears to be concluding.

(8) Ephrussi (1933) reports the loss of almost all the carbohydrate in the egg between his 12- and 40-hour tests, and very little loss in the first 12 hours. This suggests that carbohydrate is the fuel source drawn upon in later stages, possibly by about 24 hours. The embryo is perhaps using up its last reserves of food and consequently entering upon a period of starvation, pending the development of its digestive system to a point where food can be taken from the sea.

(9) Hence the apparent sources of energy in succession are: fat, non-animo nitrogen, amino nitrogen, fat, carbohydrate. More detailed information may move carbohydrate to the second or third last place.

The assignment made above of cyanide-sensitive enzyme to glycerol (or a product of it), and of cyanide-insensitive enzyme to fatty acid, is based not only on the relative abundance of the substrates, but also on the work of Emerson (1927), which indicates that cyanide-sensitive respiration results from the oxidation of carbohydrate.

Lindahl and Öhman (1936) have recently placed on some of the results cited above an interpretation differing from that given here. They say, "We see a useful conception of these relationships in the assumption that two different substrates are burnt, of which one has a small, the other a high degree of reactivity to the oxidizing 'carrier.' Shortly after fertilization only the first substrate is present in large quantities. The unchanging and small concentration of the 'carrier' determines the constant and small intensity of oxidation of this substrate. This reaction corresponds to the non-growing part of respiration, which can be increased to a marked degree by dimethylparaphenylene diamine and pyocyanine. The 'carrier' is here the limiting factor. The other substrate is very rapidly oxidized and hence does not accumulate. The rapidity of formation of this substrate determines the oxygen consumption, and is therefore the limiting factor. This is the 'growing part' of respiration, which can be inhibited by lithium." It will be seen that Lindahl and Öhman differ from the views expressed in this paper in that they: (*a*) postulate two substrates but only one enzyme system; (*b*) express no view as to the nature of the substrates; (*c*) consider that dimethylparaphenylene diamine and pyocyanine act on the same part of respiration; (*d*) state that the cyanide-sensitive part of respiration is limited by the low degree of reactivity of the substrate (and not by its scarcity); (*e*) state that the cyanide-insensitive part of respiration is limited by the scarcity of the substrate (and not by geographical segregation).

Sterol

The values for sterol will be found in column 3 of Table I. It amounts to some 0.41–0.43 mg. per million eggs, and no fluctuation was found in its concentration during the period investigated. The only comment necessary is that this particular one-tenth of the fat is not a source of embryonic energy.

Phospholipid

The results of phospholipid determinations are given in column 4. They vary from less than 1 mg. to nearly 3.5 mg. per million eggs. The values unfortunately fluctuate so much that attempts to discover a developmental trend by plotting them on a graph are unconvincing. No utilization of phospholipid can be said to be demonstrated, although the data are not sufficiently extensive to exclude the possibility. The average of all the phospholipid results is 2.17 ± 0.24 mg. per million eggs or 38 ± 4 per cent of the total fat in the unfertilized egg.

Previous Analyses

McClendon (1909) found that the total fat (ether extract) of unfertilized *Arbacia* eggs was 2.254 per cent. Harvey (1932) gives the data necessary to convert this to mg. per million as follows:

$$\text{Mg. per million eggs} = \frac{10^6 \times \text{vol. of 1 egg in cu. mm.} \times \text{egg density} \times 2.254}{100}$$

$$= 2.12 \times 1.09 \times 2.254$$

$$= 5.21 \text{ mg.}$$

McClendon also estimated the phosphorus content of his ether extract and found it to be 0.06914 per cent of the whole egg. Assuming that all the phosphorus is in the phospholipids and that it comprises 4 per cent of these (as it does in lecithin) then this figure too can be made comparable.

$$\frac{2.12 \times 1.09 \times 0.06914 \times 100}{4}$$

$$= 3.84 \text{ mg. phospholipids per million eggs.}$$

If it is valid to apply these calculations to McClendon's results then it follows that they agree in a general way with those given above.

Page (1927) has also made analyses of the fatty constituents of the unfertilized *Arbacia* egg. He states that 8.3 grams of oil were obtained from 183 million eggs by means of alcohol-ether extraction. This is in the terminology of this paper, 45.4 mg. per million eggs, or

ten times as great as McClendon's figures and those given here. A calculation of the mass of an egg from the volume and density values in Harvey shows that, according to Page, 20 per cent of the egg is fat. Now since the total solids (McClendon) only comprise 20 per cent of the egg and these are obviously not all fat, Page's results are difficult to interpret, particularly in view of his statement that *Arbacia* contains much less oil then *Asterias*. Page also precipitated the phospholipid from his extract with acetone, and his results work out at 8.4 mg. per million as against McClendon's 3.84 mg. and the 2.17 mg. above. Finally Page distilled the volatile fatty acids from his extract with steam and titrated them with NaOH, finding 9.4 mg. per million eggs. The discrepancy between Page's results and the others may lie in part in his method of counting eggs, no mention of which is made in his paper. This difficulty would not invalidate his estimates of the saponification value and his volatile acid determinations on which a part of the general argument in this paper depends.

Summary

Periodical estimations were made of the total fat, sterol and phospholipid during the first 40 hours of development of *Arbacia*. The total fat decreases up to the time of hatching (8 hours), then increases for some 10 hours, and later decreases again. The sterol concentration remains unchanged throughout the period studied. Owing to fluctuations in the phospholipid readings, a definite conclusion could not be drawn as to whether this material is utilized as a source of embryonic energy. The total fat in a million unfertilized eggs is 5.65 mg. of which 7.5 per cent is sterol and 38 per cent phospholipid. A general hypothesis of the chemistry of early sea urchin development is presented.

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